Intercellular Adhesion Molecule-1 Suppression in Skin by Topical Delivery of Anti-Sense Oligonucleotides

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We topically applied 20 nucleotide phosphorothioate intercellular adhesion molecule-1 anti-sense oligodeoxynucleotide in a cream formulation. It effectively inhibited tumor necrosis factor-α-induced expression of intercellular adhesion molecule-1 in human skin transplanted on severe compromised immunodeficient mice. The effects were concentration dependent, sequence specific, and resulted from reduction of intercellular adhesion molecule-1 mRNA levels in the skin. Intravenous administration of the drug did not show pharmacologic effects, probably due to insufficient drug concentrations in skin. Topical delivery, however, produced a rapid and a significantly higher accumulation of oligodeoxynucleotide in the epidermis and dermis. The results strongly suggest that topically applied anti-sense oligonucleotides can be delivered to target sites in the skin and may be of considerable value in the treatment of psoriasis and other inflammatory skin disorders. ***Key words: drug delivery/psoriasis. J Invest Dermatol 115:805–812, 2000***

Psoriasis is a multifactorial disease of unknown etiology affecting approximately 2% of the population. Psoriasis is characterized by epidermal hyperplasia, dermal angiogenesis, epidermal influx of polymorphonuclear leukocytes, and the presence of mononuclear cells in the papillary dermis and in the epidermis. Initially, the disease was thought to be due to a dysregulation of keratinocyte proliferation; however, current views hold that defects in T cell regulation are primarily responsible for the disease (Bos and De Rie, 1999). This view is supported by a number of lines of evidence, including the observation that the mononuclear cell infiltrate present at the dermal–epidermal junction consists primarily of activated T cells and antigen-presenting cells and precedes epidermal proliferation. Secondly, transplantation of allogeneic bone marrow from a psoriatic patient appeared to have transmitted psoriasis to the recipient (Gardembas-Pain *et al*, 1990). Thirdly, injection of activated mononuclear cells from psoriatic patients into normal human skin orthotopically transplanted on to severe compromised immune deficient (SCID) mice resulted in a psoriatic-like phenotype (Wrone-Smith and Nickoloff, 1996; Nickoloff *et al*, 1999; Nickoloff and Wrone-Smith, 1999). Fourthly, numerous studies in psoriasis have documented increased expression of T cell cytokines and adhesion molecules that recruit T cells to sites of inflammation (Nickoloff, 1991). Finally, clinical studies of agents that directly affect T cell function such as cyclosporine A, interleukin-2 toxin, and CTLA4-immunoglobulin appear to be effective in reducing the severity of the disease (Mueller and Herrmann, 1979; Finzi *et al*, 1993; Gottlieb *et al*, 1995; Abrams *et al*, 1999).

As current therapies for psoriasis are felt to be unsatisfactory for patients with moderate to severe disease, there are a number of experimental approaches being investigated for the treatment of this patient population (Greaves and Weinstein, 1995; Griffiths, 1998). One such approach is to inhibit leukocyte infiltration into the skin through the regulation of leukocyte–endothelial cell adhesion molecule function. Molecules such as E-selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular adhesion molecule-1 expressed on the activated vascular endothelium are required for the recruitment of lymphocytes into the skin (Picker *et al*, 1991; Scheynius *et al*, 1993; Springer, 1994). ICAM-1, a member of the immunoglobulin gene superfamily, is expressed at low levels on resting endothelial cells, and is markedly upregulated on endothelial cells in response to inflammatory cytokines such as interleukin 1 and tumor necrosis factor (TNF). ICAM-1 is also expressed on activated antigen-presenting cells and inflamed stromal and epithelial cells. Consistent with its expression pattern on nonendothelial cells, ICAM-1 also contributes to leukocyte activation in inflamed tissue by providing costimulatory signals (Altmann *et al*, 1989; Nickoloff *et al*, 1993; Damle *et al*, 1994; Schnitzler *et al*, 1999). In human psoriatic plaques, ICAM-1 expression is markedly upregulated on endothelial cells, dermal fibroblasts, and keratinocytes, whereas normal skin expresses low levels of ICAM-1 only on endothelial cells (Griffiths *et al*, 1989; Lisby *et al*, 1989; Das *et al*, 1994; Paukkonen *et al*, 1995). The finding that ICAM-1 expression is markedly increased in human psoriatic plaques, and the known part ICAM-1 plays in leukocyte recruitment to sites of inflammation and in leukocyte activation, suggest that it may be an attractive molecular target for psoriasis treatment. Therefore, several therapeutic strategies are being pursued to interfere with ICAM-1 function.

Selective regulation of gene expression by anti-sense oligonucleotides has been extensively explored as a research tool and more recently as therapeutic agents (Crooke and Bennett, 1996;

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Abbreviations: CGE, capillary gel electrophoresis; ICAM-1, intercellular adhesion molecule-1; ODN, anti-sense oligodeoxynucleotide; SCID, severe compromised immunodeficient.

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Bennett, 1999). We have identified an anti-sense oligodeoxynucleotide (ODN), ISIS 2302, that selectively inhibits human ICAM-1 expression in a variety of cell types by an anti-sense mechanism of action (Bennett *et al*, 1994; Miele *et al*, 1994; Nestle *et al*, 1994). Systemically administered ISIS 2302 was found to reduce ICAM-1 expression in human skin transplanted on to SCID mice and partially block heterologous T lymphocytes from migrating into the epidermis when injected in the dermis of the graft in a model mimicking lichen planus (Christofidou-Solomidou *et al*, 1997). Murine and rat homologs of ISIS 2302 have been used in a variety of rodent inflammatory models to demonstrate the beneficial effects of inhibiting ICAM-1 expression (Stepkowski *et al*, 1994, 1998; Kumasaka *et al*, 1996; Bennett *et al*, 1997). ISIS 2302 is currently in advanced clinical trials for the treatment of Crohn's disease, having shown encouraging activity in a pilot study (Yacyshyn *et al*, 1998), and is being investigated in additional pilot studies for the therapy of inflammatory diseases, including psoriasis (Bennett, 1999).

Currently, all therapeutic anti-sense oligonucleotides in development, including ISIS 2302, are being investigated as parenteral products. For life-threatening or severely debilitating diseases, parenteral administration of drugs is well accepted; however, for less severe diseases a more convenient route of administration would be desirable. To this end we have investigated the feasibility of topical delivery of anti-sense oligonucleotides. Using an optimized formulation, we have found that topical delivery of phosphorothioate oligodeoxynucleotides appears to be feasible. We demonstrate that compared with intravenous administration, topical application of an oligonucleotide results in approximately 4000-fold more intact oligonucleotide in epidermis and 150-fold more in dermis. In addition we demonstrate a sequence-specific reduction in ICAM-1 expression in mouse skin and orthotopic human skin grafts following treatment with topically applied ICAM-1 anti-sense oligonucleotide. This is the first study demonstrating specific reduction in gene expression following topical application of an anti-sense oligonucleotide.

MATERIALS AND METHODS

Oligonucleotide selection and formulation All the oligonucleotides used in these studies were phosphorothioate oligodeoxynucleotides. Synthesis and purification was performed as previously described (Chiang *et al*, 1991). **Table I** lists the sequences and functions of ODN used. The ICAM-1 ODN have been thoroughly characterized for pharmacologic effects (Bennett *et al*, 1994; Bennett, 1998). ISIS 2503 was used as a marker for ODN distribution as it is easily recognized by a specific antibody as described in the "Histopathology and oligonucleotide detection" section of *Materials and Methods*. A cream with the following composition was used in the studies: glyceryl monostearate (10%), hydroxypropyl methylcellulose (0.5%), isopropyl myristate (10%), methylparaben (0.5%), propylparaben (0.5%), polyoxyl-40-stearate (15%), and water.

ODN distribution in transplanted human skin

Human skin xenograft preparation An orthotopic human skin graft model was used for the distribution and pharmacologic evaluation of ODN formulations (Yan *et al*, 1993). Human skin was obtained from cadaver or from plastic surgery donors after informed consent and placed in Dulbecco's minimal Eagle's medium (Gibco BRL, Grand Island, NY) with 10% heat-inactivated fetal bovine serum (Gibco BRL) and 100 IU penicillin-streptomycin per ml (Gibco BRL) overnight. Cadaver skin was received keratomed, and surgery resected skin was cut by hand to 0.5 cm thickness. All skin samples were cut into 2×2 cm pieces and orthotopically transplanted on to 6–8 wk old ICR SCID mice (Taconic, Germantown, NY). The mice were prepared by removing a 1.5×1.5 cm piece of skin on the right flank. The human skin was then xenografted on this area by suturing with 4–0 silk or stapling using sterile 9 mm wound clips. The skin was covered with a nonadhering dressing and then covered with 2½ inch adhesive bandages stuck together and then wrapped around the mouse. After 1 wk, dressing and staples were removed. Within 4–6 wk there was full engraftment without any signs of inflammation or rejection. All mice were tested for IgG by radial immunodiffusion (The Binding Site, San Diego, CA) before being assigned to a study.

ODN administration Twelve mice with grafted human skin were divided in four study groups. Each group received a dose of ISIS 2503 via either intravenous, subcutaneous, intradermal or topical route of delivery. ISIS 2503 was chosen for these distribution studies as it has multiple phosphorothioate CG or TCG motifs for which the 2E1-B5 IgG1 has a strong affinity (see "Histologic evaluation" section for details).

Intravenous and subcutaneous administration was performed using 330 μl (average body weight 33 g) and 360 μl (average body weight 36 g) solution of 0.5 mg ISIS 2503 per ml in normal saline. Intradermal administration was performed using 40 μl of a 5 mg ISIS 2503 solution per ml (average body weight = 27 g). For topical application, 200 μl of 20 mg ODN per ml in cream was placed in plastic holders and placed on a 2 cm^2 patch the transplanted skin with 2½ inch adhesive bandages stuck together and then wrapped around the mouse and left in place for 24 h. The amount of cream used was necessary to fill the holders and cover the skin area with a 1–2 mm layer of cream.

Histologic evaluation Skin samples were carefully washed with 2% Tween 20 in phosphate-buffered saline to remove any excess cream and fixed in 10% neutral-buffered formalin for 24 h before transferring to 70% ethanol for dehydration and storage. The tissues were embedded in paraffin and sectioned at 4 μm for analysis. The sections were deparaffinized in xylene and hydrated through graded alcohols for ODN immunostaining and hematoxylin and eosin stains. The affinity-purified antibody used in this work, 2E1-B5 (Berkeley Antibody Company, Berkeley, CA), is an IgG1 that specifically recognizes a CG or TCG motif in phosphorothioate oligonucleotides. Endogenous peroxidase activity was quenched with peroxidase blocking reagent (Dako Corporation, Carpenteria, CA) for 10 min; sections were rinsed with phosphate-buffered saline and treated with proteinase K (Dako) for 20 min. After blocking with normal donkey serum (Jackson Laboratories, Burlingham, AB), sections were incubated for 1 h with 2E1 monoclonal antibody diluted at 1:1000. After rinsing, the antibody was detected using horseradish peroxidase-donkey anti-mouse IgG F(ab)′2 diluted 1:100 (Jackson) for 1 h. 3,3′-diaminobenzidene (Dako) was used as a substrate. The tissue sections were counterstained with hematoxylin, dehydrated, and mounted with coverslips. Serial sections of the tissues were stained with hematoxylin and eosin for routine histopathologic analysis.

Tissue sections were blinded and ISIS 2503 immunostaining was evaluated using a Zeiss Axiolab microscope with a Sony color video camera at various magnifications up to 200×.

ODN distribution in hairless mouse skin Two groups of three hairless mice were administered nonexchangeable tritium-labeled ISIS 2302 either as an intravenous injection (2 mg per kg ISIS 2302 in saline) or as a cream (2% ISIS 2302 in cream). ^{3}H-thymidine was placed seven bases remote from the 3′ end. For topical application, 100 mg cream was applied on a 4 cm^2 skin surface, covered with a Teflon patch and kept in place with an adhesive bandage. At the end of 24 h, the mice were killed by carbon dioxide asphyxiation. The skin was thoroughly rinsed with mild surfactant solution (2% Tween 80 in water) to remove surface bound radioactivity. Skin samples were collected from the application site of the topical dose and

Table I. Sequences and functions of oligonucleotides

ODN	Sequence	Function
ISIS 2302	5′-GCCCAAGCTGGCATCCGTCA-3′	human ICAM-1 anti-sense
ISIS 8424	5′-GACGCATCGCGCCTACATCG-3′	10-base mismatch of ISIS 2302
ISIS 3082	5′-TGCATCCCCCAGGCCACCAT-3′	murine ICAM-1 anti-sense
ISIS 8997	5′-TCGCATCGACCCGCCCACTA-3′	12-base mismatch of ISIS 3082
ISIS 2503	5′-TCCGTCATCGCTCCTCAGGG-3′	for immunohistochemical localization of ODN

a similar site from the mice administered with intravenous ISIS 2302. The epidermis and stratum corneum were mechanically separated using a scalpel from the remaining skin. Removal of the epidermis was confirmed by histology. Subcutaneous fat layers were also removed from the skin samples. Samples were weighed and digested in Soluene (Packard Instrument, Meriden, CT) overnight, neutralized with hydrochloric acid, and counted for radioactivity using a LS6500 scintillation counter (Beckman Coulter, Fullerton, CA).

Analysis of ISIS 2302 in skin by capillary gel electrophoresis (CGE) A sample of whole skin was also used for the analysis of ISIS 2302 by CGE. ISIS 2302 and its metabolites were isolated from skin samples using a phenol/chloroform extraction followed by one-step solid phase extraction as described previously (Yu *et al*, 1999). An appropriate amount of internal standard (T_{27}, a 27-mer-phosphorothioate oligodeoxythymidine) was added to each sample prior to extraction. Extracted tissue samples were analyzed by CGE using a Beckman P/ACE Model 5010 capillary electrophoresis instrument (Beckman Coulter). The procedure had been validated for quantitative analysis of ISIS 2302 and its chain-shortened metabolites (formed by removal of one, two, or more nucleotides from the parent compound). The concentrations obtained by radioactivity were corrected for full-length ISIS 2302 content using the CGE profile. The results are presented as total ISIS 2302 and full-length ISIS 2302.

Skin pharmacokinetics of ODN in rats and pigs

Study design and administration of ODN to rats Sprague–Dawley (Harlan Sprague Dawley, Indianapolis, IN) male rats were treated with approximately 200 mg of 2% ISIS 2302 cream. The cream was spread over an area of approximately 12 cm^2 on the dorsal surface (17 mg cream or 0.34 mg ISIS 2302 per cm^2 skin). The application sites were covered with a Teflon patch and held in place using an elastic wrap bandage. The treatment sites were kept occluded for 6 h. At the end of occlusion period, application sites were cleaned of residual cream with a mild surfactant (2% Tween 80 in water) and remained unocclused for the remainder of the study. Three rats were killed by exsanguination under isoflurane anesthesia per time point. Skin samples collected, frozen on dry ice and stored at –70°C until analyzed for ISIS 2302 concentration.

Study design and administration of ODN to pigs Two adult Yucatan minipigs (one male and one female) (Sinclair Research Center, Columbia, MO) were administered approximately 0.5 g of 2% ISIS 2302 cream on eight patches of 20 cm^2 surface area on the right side (25 mg cream or 0.5 mg ISIS 2302 per cm^2 skin). The application sites were covered with a Teflon patch and held in place using an adhesive bandage. The treatment sites were kept occluded for up to 6 h. At the end of occlusion period, application sites were cleaned of residual cream with a mild surfactant (2% Tween 80 in water) and remained unocclused for the remainder of the study. Dosing was performed sequentially on eight sites starting with 96 h before sample collection to 1 h before collection. The animals were killed with a lethal dose of Beuthanasia (Schering-Plough Animal Health, Kenilworth, NJ) at the end of the study. Skin samples collected, frozen on dry ice, and stored at –70°C until analyzed for ISIS 2302 concentration.

Analysis of ISIS 2302 concentration in skin The analysis of ISIS 2302 concentration was performed as described above for ODN distribution in hairless mouse skin. The results are presented as the concentration of intact ISIS 2302 as well as a sum of ISIS 2302 and all its chain-shortened metabolites.

ICAM-1 suppression in hairless mouse skin

ODN administration and ICAM-1 induction in skin Topical application of cream was performed by placing 200 µl of cream into a plastic reservoir attached to a ½ inch adhesive bandage and placed on to the xenografted skin. Application of cream was performed at –48, –24, and –4 h prior to phorbol ester induction. Induction was performed with 20 µl of 2 mg ml phorbol myristate acetate (Sigma, St Louis, MO) solution in acetone. Mice were killed by carbon dioxide asphyxiation 4 h after phorbol ester induction. Skin samples were collected for immunohistochemical localization of ICAM-1 protein and for mRNA analysis as described below.

ICAM-1 immunohistochemistry A section of skin sample was embedded in OCT compound (Sakura, Japan) and snap frozen in isopentane (2-methylbutane, J.T. Baker, Phillipsburgh, NJ) that was previously cooled with dry ice. Four micron thick cryostat tissue sections were fixed in ice-cold acetone and stained with mouse monoclonal antibodies to human ICAM-1 (Pharmingen, San Diego, CA) at 15 µg per ml. The primary antibodies were detected with horseradish peroxidase conjugated donkey anti-mouse IgG F(ab′)2 that was absorbed against multiple species (Jackson Laboratories, West Grove, PA). All slides were stained on the Dako Autostainer (Dako) and 3,3′-diaminobenzidene was used as a substrate (Dako). The sections were counterstained with hematoxylin, dehydrated, and mounted with permanent mounting media for evaluation. The samples were then evaluated using a Zeiss Axiolab microscope with a Sony color video camera at various magnifications up to 200×.

RNA isolation and analysis A section of skin sample was homogenized in 4 M guanidinium isothiocyanate and total cellular RNA was isolated by CsCl gradient centrifugation (Chirgwin *et al*, 1979). RNA was separated on a 1% agarose gel containing 1.1% formaldehyde, then transferred to a nylon membrane and ultraviolet cross-linked to the membrane using a Stratagene ultraviolet cross-linker 2400. Blots were hybridized with cDNA probes that were random primed (Prime-a-Gene, Promega, Madison, WI) and purified on NAP-5 columns (Pharmacia, Peapack, NJ) followed by hybridization for 1–2 h in QuikHyb solution (Stratagene, San Diego, CA). Blots were washed twice at 25°C in 2 × sodium citrate/chloride buffer with 0.1% sodium dodecyl sulfate for 10 min each and then washed one time in 0.1% sodium citrate/chloride buffer with 0.1% sodium dodecyl sulfate at 60°C for 20 min. Hybridizing bands were visualized and quantitated using a Molecular Dynamics PhosphorImager. The blots were stripped by pouring boiling 0.1% sodium citrate/chloride buffer and 0.1% sodium dodecyl sulfate solution on the blots and incubating under gentle agitation for 5 min. Blots were reprobed with G3PDH (Clontech, Palo Alto, CA) to confirm equal RNA loading.

ICAM-1 suppression in human skin

Human skin xenograft preparation Skin xenograft preparation was the same as that described for ''ODN distribution in transplanted human skin'' earlier in this section.

ODN administration and ICAM-1 induction in human skin xenografts Topical application of cream was performed by placing 100 µl of cream on to a Teflon patch attached to a ½ inch adhesive bandage which was placed on to the xenografted skin. Application of cream was performed at –48, –24, and –4 h prior to TNF-α induction. Induction was performed with 4000 units recombinant human TNF-α (R&D Systems, Minneapolis, MN) mixed with colloidal carbon in a 20 µl volume by intradermal injection using a 27 G ½ inch needle attached to a 100 µl Hamilton syringe. Mice were killed by carbon dioxide asphyxiation 24 h after TNF-α induction for histologic examination.

ICAM-1 immunohistochemistry ICAM-1 protein localization by immunohistochemistry was performed as described for ICAM-1 suppression in hairless mouse skin with the following exception: the antibody used was a mouse monoclonal antibodies to human ICAM-1 (Pharmingen, San Diego, CA) at 15 µg per ml. The primary antibodies were detected with horseradish peroxidase-conjugated donkey anti-mouse IgG F(ab′)2 that was absorbed against multiple species (Jackson Laboratories, West Grove, PA).

Tissue sections were blinded and ICAM-1 immunostaining was evaluated using a Zeiss Axiolab microscope with a Sony color video camera. Image analysis on the dermis and epidermis was performed separately using Image Pro Plus (Media Cybernetics, Silver Springs, MD) at a magnification of 200×. Data were collected in five fields and expressed as the percentage of the total area that was positively stained.

RESULTS

Topical ODN distributes to epidermis and dermis, injected ODN distributes primarily in dermis in transplanted human skin Intravenous and subcutaneous administration of ISIS 2503 to SCID mice with human skin grafts resulted in an accumulation of ISIS 2503 only in the papillary dermis, with no visible staining in the reticular dermis or epidermal keratinocytes (**Figs 1*a*, *b***). Intradermal administration resulted in some accumulation in the cytoplasm of keratinocytes directly above the site of dermal injection (**Fig 1*c***). Topical delivery resulted in accumulation of ODN in epidermal keratinocyte nuclei and accumulation in fibroblasts (predominantly in the cytoplasm) throughout the dermis (**Fig 1*d***).

Topical ODN dosing results in significantly higher accumulation in epidermis and dermis compared with intravenous dosing in hairless mouse skin ISIS 2302 was injected intravenously at a dose of 2 mg per kg or applied on the skin at a dose of 0.5 mg per cm^2. The group administered topical ODN showed 1536-fold more total ODN in epidermis and 54-

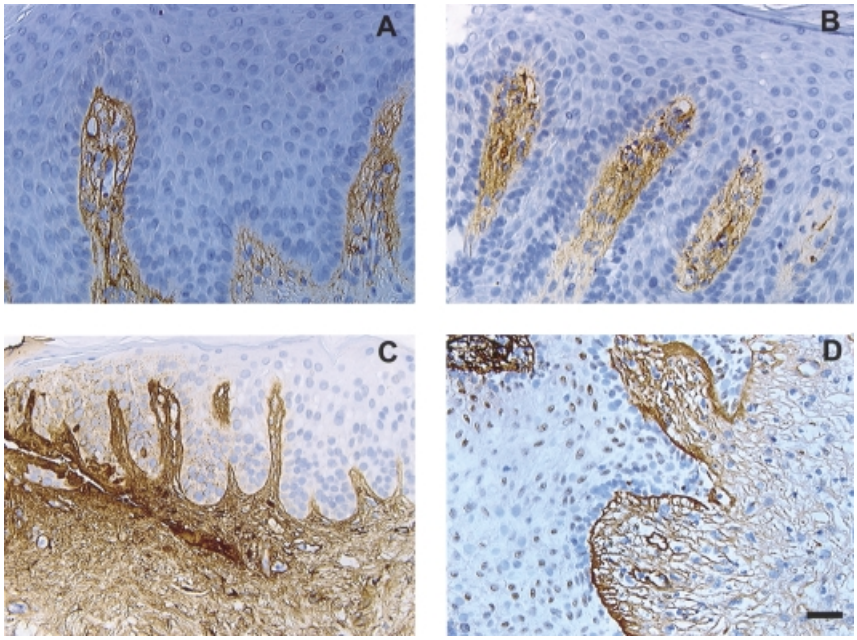


Figure 1. Topical ODN distributes to epidermis and dermis, injected ODN distributes in dermis. (*a*) Intravenous injection: ODN staining is visible in the dermal folds with no staining in the epidermis. (*b*) Subcutaneous injection: ODN staining is visible in the dermal folds with no staining in the epidermis. (*c*) Intradermal injection: diagonal needle track is clearly visible in the section. A large amount of ODN is deposited in the dermis surrounding the point of injection; however, low staining in the epidermis directly above the needle track indicates poor migration of ODN from dermis to epidermis. (*d*) Topical application: staining of epidermal keratinocyte nuclei is clearly seen along with significant cytoplasmic distribution in the dermis. *Scale bar*: 20 μm.

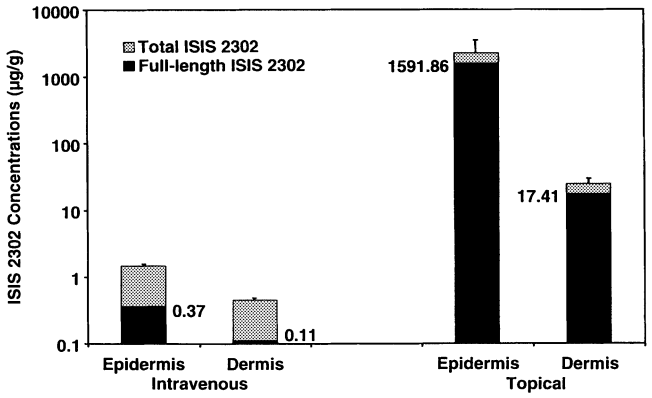


Figure 2. Topical dosing delivers significantly greater amount of ODN to skin when compared with intravenous dosing. The combined height of the open and solid bar represent the total ISIS 2302 concentration and the solid bar and the numbers next to the solid bar show the amount of full-length ISIS 2302 in epidermis and dermis after intravenous and topical administration to hairless mice. Topical dosing delivered 4300-fold greater full-length ISIS 2302 to epidermis and 156-fold greater full-length ISIS 2302 to dermis than intravenous dosing at 2 mg per kg (n = 3, mean ± SD).

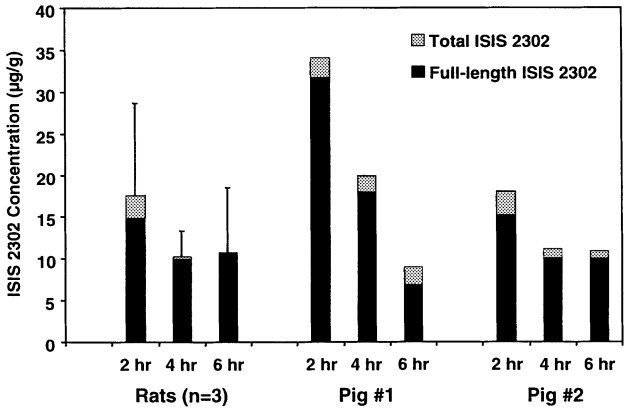


Figure 3. Rapid uptake and extended residence of ODN in skin. Rapid uptake of ISIS 2302 is seen in the skin of rats and pigs after topical application. Bars show total ISIS 2302 and full-length ISIS 2302 at three time points in three rats and two pigs. Both the species show slow elimination and very little metabolism. The numbers for the rat study represents mean and standard deviation and numbers for the pig represent the individual values.

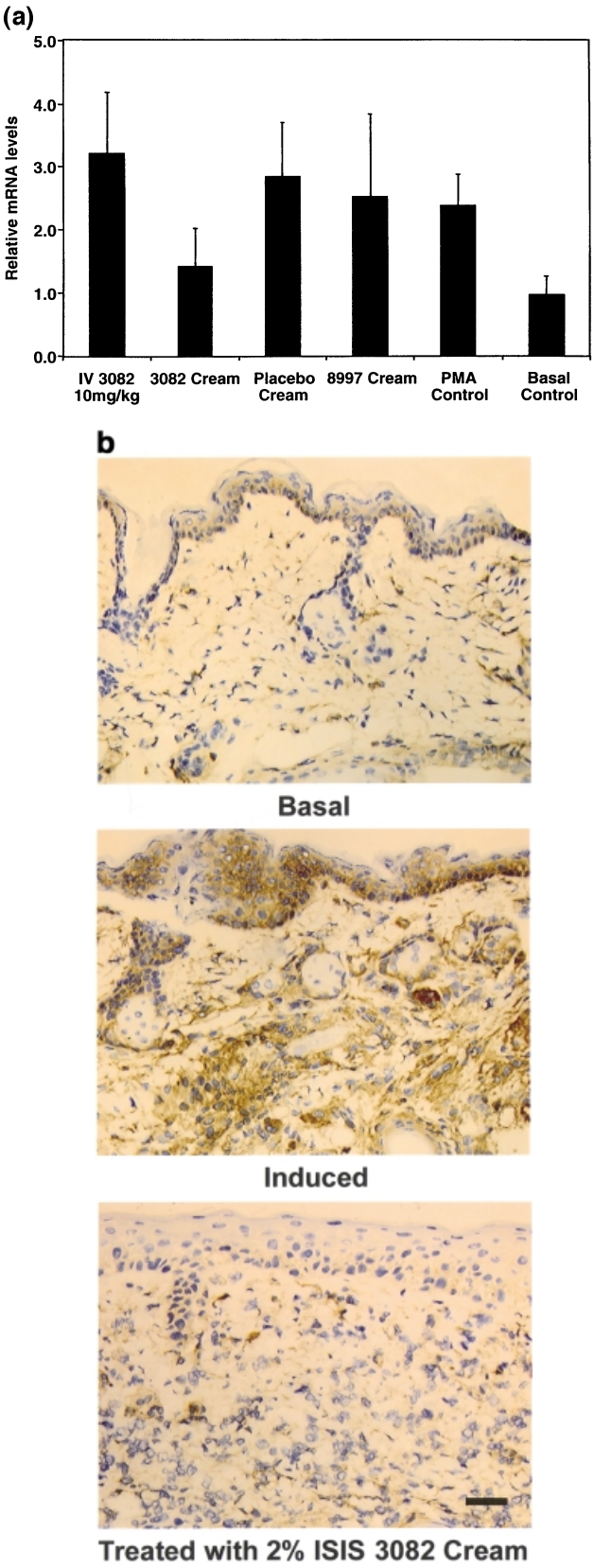
fold more total ODN in dermis than the intravenous-treated group (**Fig 2**). Topical dosing also resulted in lower metabolism of ISIS 2302 in the skin. After 24 h, the full-length fraction of ISIS 2302 was 71% as compared with 25% after intravenous dosing. When corrected for the full-length fraction of ISIS 2302, the group administered topical ODN showed 4300-fold more ISIS 2302 in epidermis and 158-fold more ISIS 2302 in dermis than the intravenous group.

Although the skin sample for epidermal quantitation contained stratum corneum, several experiments conducted to determine localization of ODN in skin using ISIS 2503 by immunohistochemistry showed minimal localization of ODN in stratum corneum. The high concentrations in this study can be attributed primarily to ODN concentrations in the epidermal region below stratum corneum. Attempts to separate stratum corneum by tape stripping were unsuccessful. Tape stripping the hydrated skin usually resulted in patchy removal of epidermis rather than just stratum corneum. Separation of epidermis from skin was also observed by other investigators working with hydrated skin (Noleen *et al*, 1994; Ocheltree, 1996).

Rapid uptake and extended residence of ODN in skin Kinetics of ODN uptake in the skin from the cream formulation were studied in rats and pigs. Both species showed a rapid uptake of ODN after topical administration with skin concentrations reaching the 20–30 μg per g range within 2 h. The levels remained high (about 10 μg per g) at the 6 and 24 h time point. Analysis of ODN by CGE indicated very little metabolism of the accumulated ODN remaining intact in the skin through 24 h in both species (**Fig 3**). Whereas the number of animals used in this study is limited, additional pharmacokinetic studies in rats, pigs, and humans with ISIS 2302 and other ODN sequences are underway.

Demonstration of specific reduction in ICAM-1 expression following topical administration of an anti-sense oligonucleotide To demonstrate that topical application of an antisense oligonucleotide resulted in functional delivery to the skin, we evaluated the effects of a phosphorothioate oligodeoxynucleotide targeting murine ICAM-1, ISIS 3082, in a model of skin

inflammation initiated by topical phorbol ester administration. Phorbol ester promoted a 2.5-fold increase in ICAM-1 mRNA in skin 4 h after application. Pretreatment of mice with 10 mg per kg ISIS 3082 by intravenous administration failed to prevent the upregulation of ICAM-1 mRNA in skin; however, topical application of a 2% formulation of ISIS 3082 reduced ICAM-1 mRNA by approximately 66% (**Fig 4*a***). The control oligonucleotide, ISIS 8997, and the vehicle cream failed to reduce ICAM-1 mRNA. These results demonstrate that topical application of the ICAM-1 oligonucleotide resulted in sequence-specific reduction in ICAM-1 expression in mouse skin. The results were confirmed by immunohistochemical localization of ICAM-1 protein in skin (**Fig 4*b***). The results show a reduction in ICAM-1 protein to baseline levels in epidermis and dermis after treatment with ISIS 3082 cream.



The architecture of mouse skin differs from that of human skin, in particular the epidermis is significantly thinner than in human skin. Therefore, to demonstrate that the formulation also would deliver anti-sense oligonucleotide to the appropriate sites in human skin, we used the orthotopic human skin graft model. ICAM-1 expression was induced by direct injection of TNF-α into the skin. Mice were dosed three times with cream containing 0–2% ISIS 2302 prior to TNF-α injection. Injection of TNF-α into skin promoted a marked upregulation of ICAM-1 expression in the human skin, particularly in keratinocytes in the basal layer (**Fig 5*a***, ***b***). ISIS 2302 reduced ICAM-1 expression in a concentration-dependent manner, with maximal activity observed between 0.5 and 2% (**Fig 5*a***, ***b***). The oligonucleotide reduced ICAM-1 expression in both the epidermis and dermis. ISIS 2302 at a concentration of 0.1% reduced ICAM-1 expression to a lesser extent. The control oligonucleotide, ISIS 8424 did not significantly reduce ICAM-1 expression (**Fig 5**).

DISCUSSION

We have shown for the first time that anti-sense phosphorothioate oligodeoxynucleotides can be used to inhibit expression of ICAM-1 in epidermis and dermis when applied topically in a cream formulation. The results show that ISIS 2302 and its murine analog, ISIS 3082, inhibit ICAM-1 induction in human and mouse skin, respectively. The pharmacologic effect was also shown to be highly sequence-specific and was the result of ICAM-1 mRNA suppression. ISIS 2302 and ISIS 3082 are designed specifically to inhibit the expression of ICAM-1 by hybridization with the 3′-untranslated region of ICAM-1 mRNA (Chiang *et al*, 1991). The resulting RNA–DNA duplex undergoes a RNase-mediated hydrolysis that causes a reduction in ICAM-1 protein (Bennett *et al*, 1994; Bennett, 1998). Intradermal injection of ODN into human skin xenografts resulted in extensive cytoplasmic accumulation in dermal fibroblasts, but minimal accumulation in epidermal keratinocytes (**Fig 1*c***). Subcutaneous and intravenous injection showed cytoplasmic accumulation in the dermis but not in the epidermis (**Fig 1*a***, ***b***). In a recent study, it was proposed that topical anti-sense therapy could be tested by the use of intradermal injection into human skin xenografts on mice (White *et al*, 1999). The results from the present study also suggest that high doses of intradermal ODN would be required to achieve measurable levels of ODN in the epidermis. This suggests that, when transporting ODN from dermis to epidermis, very high concentrations of ODN are required in dermis before measurable accumulation occurs in

Figure 4. Anti-ICAM-1 ODN suppresses ICAM-1 mRNA in mice. (*a*) ICAM-1 mRNA levels in mice skin treated with anti-ICAM-1 ODN: only the group treated with topical ISIS 3082 cream showed a lowering of ICAM-1 mRNA ($p = 0.01$ against intravenous and $p = 0.04$ against vehicle cream, Student's t test) to a level that was not different from basal level. When compared with phorbol myrisate acetate control, intravenous dosing of ISIS 3082, vehicle cream, and cream with ISIS 8997 (a scrambled control sequence of ISIS 3082) showed no reduction in ICAM-1 mRNA. All the comparisons were made in one experiment with three animals per group (mean ± SD). (*b*) Photomicrograph showing immunohistochemical localization of ICAM-1 protein in mouse skin confirms the suppression of ICAM-1 protein in mice treated with ISIS 3082. Three animals were evaluated for each group, the micrographs show a representative section from one animal. *Scale bar*: 20 μm.

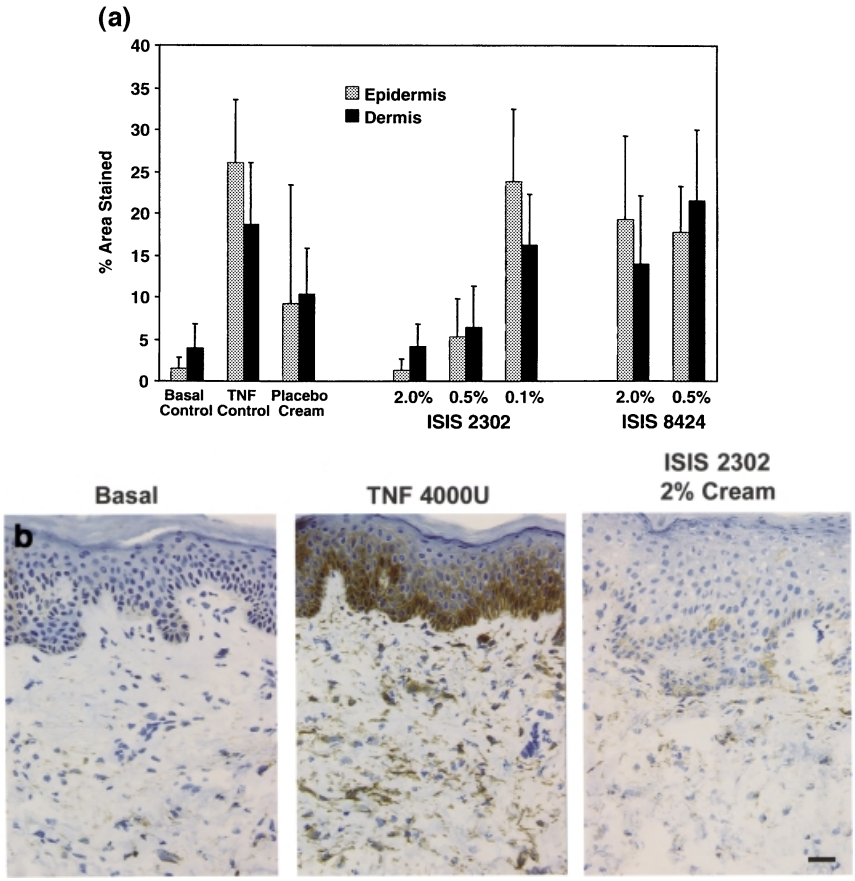


Figure 5. Concentration-dependent suppression ICAM-1 expression in skin. (*a*) Percentage of area stained by anti-ICAM-1 antibody in sections of skin treated with various formulations prior to the induction of ICAM-1 by TNF-α. Only the groups treated with topical ISIS 2302 at 2% ($p < 0.00001$ for epidermis and remaining skin, Fisher's protected least significant difference) and 0.5% ($p < 0.00001$ for epidermis and $p = 0.0002$ remaining skin, Fisher's protected least significant difference) dose strengths showed a significant lowering of ICAM-1 close to the basal levels. A very low concentration of ISIS 2302 (0.1%) cream and cream with ISIS 8424 (a scrambled control sequence of ISIS 2302) showed little reduction in ICAM-1. The differences between epidermis and dermis were not significant in any group. All the comparisons were made in one experiment with three animals per group (mean ± SD). (*b*) Representative photomicrographs of the skin stained for ICAM-1 from the basal-, positive control-, and drug-treated groups. The group treated with 2% ISIS 2302 cream showed a complete suppression of ICAM-1 in epidermis and very little ICAM-1 staining in dermis. Three animals were evaluated for each group. *Scale bar*: 20 μm.

the epidermal keratinocytes. On the other hand, ODN applied topically in the cream formulation rapidly penetrated the skin and accumulated in the nuclei of epidermal keratinocytes and the cytoplasm of dermal fibroblasts (**Fig 1*d***).

The differences in subcellular distribution in various skin cells following topical administration can be explained on the basis of differential interaction between ODN and the cells of epidermis and dermis. Following topical ODN application in cream, keratinocytes showed a nuclear accumulation of ODN whereas cells in dermis showed a predominantly cytoplasmic accumulation (**Fig 1*d***). It was shown earlier that keratinocytes in culture take up ISIS 2302 rapidly in the absence of cationic lipids. The internalized ODN escapes the cytoplasmic vesicles and accumulates in the nuclei of keratinocytes. Cultured fibroblasts, smooth muscle cells, and endothelial cells also take up a similar or greater amount of ISIS 2302 in the absence of cationic lipids, but the distribution is restricted to cytoplasmic granules with no nuclear accumulation evident. When nuclear accumulation of ISIS 2302 was evident in cultured keratinocytes an inhibition of ICAM-1 expression could be measured (Nestle *et al*, 1994).

These differences in the distribution as a function of route of delivery dictate the ultimate concentrations of ODN achieved in the epidermis and dermis. Topical application of a 2% ODN cream resulted in a 4300-fold greater concentration in epidermis and 156-fold greater concentration in dermis than an intravenous dose at 2 mg per kg. Intravenous dosing predominantly delivers a small amount of ODN to the dermis and practically undetectable amounts to the epidermis. Thus topical delivery is superior to systemic intravenous delivery for targeting ODN to the epidermis and dermis.

Analysis of ISIS 2302 concentration in skin by CGE as a function of time showed a rapid absorption, with time to maximum concentration of less than 2 h in rat and pig skin. Removal of cream from the skin of rats and pigs after 6 h of application was followed by a slow elimination from the skin directly under the site of application. At the end of 24 h, about 50% of the maximum intact concentration of ISIS 2302 was still present in the skin directly under the site of application. Drug elimination from the site occurred principally via re-distribution into surrounding skin and absorption into the systemic circulation (data not shown). The analytical method has been validated for detecting metabolic degradation products; however, there was negligible detection of deletion sequences in CGE profiles of the drug isolated from skin after 24 h. Although only a few time points were used in this study, the skin pharmacokinetic profile indicates the possibility of prolonged pharmacologic effect from a single topical dose.

ICAM-1 is expressed at low levels on resting keratinocytes and endothelial cells, but can be markedly upregulated in response to inflammatory mediators such as TNF-α, interleukin-1, and interferon-γ (Griffiths *et al*, 1989). ISIS 2302 at 0.5% and 2% in the cream formulation applied to intact human skin reduced the levels of subsequently induced ICAM-1 in epidermis and dermis to

near baseline levels in the TNF-α-induced models of inflammation. Dosing of a 0.1% ISIS 2302 cream formulation did not produce a significant reduction in ICAM-1. Thus, a clear dose–response was seen in the pharmacologic effect of topical ISIS 2302 cream on TNF-α-induced expression of ICAM-1 in human skin, indicating that the formulation successfully delivered ISIS 2302 to intact human skin at concentrations sufficient to produce a pharmacologic effect.

Previous studies have shown that ISIS 2302 selectively inhibits cytokine-induced ICAM-1 expression in a variety of human target sites *in vivo* (Miele *et al*, 1994; Christofidou-Solomidou *et al*, 1997). A murine analog, ISIS 3082, has also been shown to be active in multiple murine models of inflammation (Stepkowski *et al*, 1994; Kumasaka *et al*, 1996; Bennett *et al*, 1997). In a recent placebo-controlled clinical study, systemic (intravenous) therapy with ISIS 2302 has demonstrated effective suppression of ICAM-1 expression in intestinal mucosa of patients with Crohn's disease (Yacyshyn *et al*, 1998). In all of these studies the ODN was administered systemically to achieve therapeutic levels at the intended site of action. Our study demonstrates the ability of a formulation to deliver ODN to the intended site of action in the skin, thereby potentially providing high local therapeutic effects with minimal effects on unintended targets.

There are several reports in the literature describing the non-anti-sense effects of phosphorothioate ODN and questioning their anti-sense mechanism of action (Wagner, 1994; Stein, 1995, 1996); however, ISIS 2302 has been shown to act by an anti-sense mechanism with multiple lines of evidence (Bennett, 1998). Our study further adds to the list of evidence with the inability of ISIS 8424, a scrambled control sequence of ISIS 2302, to prevent TNF-α-induced ICAM-1 expression in human skin at doses similar to the effective doses of ISIS 2302. In addition, the murine analog, ISIS 3082, produced a sequence-specific inhibition of ICAM-1 mRNA in mice with its scrambled control sequence, ISIS 8997, showing no ICAM-1 mRNA inhibition. This study uses the induction of ICAM-1 expression after administration of ODN. In reality, inflammation resulting in higher ICAM-1 levels would be already present when treatment is initiated. We believe that presence of inflammation will have little impact on the pharmacologic effect of ISIS 2302.

Other studies have shown mixed results with penetration of ODN in epidermis after topical and systemic administration. In one study of systemically administered ODN, little or no accumulation of ODN was seen in skin by imaging techniques (Butler *et al*, 1997) whereas in another study pharmacologic effects in skin were evident after systemic administration of ODN, although no direct measurement or localization of ODN in skin was evaluated (Christofidou-Solomidou *et al*, 1997). The differences between the two studies could be the results of a difference in the sensitivity of methods to determine ODN or its effects in skin. Other studies have used different vehicles for delivering the ODN to the skin that increase uptake by hair follicles (cationic lipids, Lieb *et al*, 1997) or chemically modified the ODN that are more lipophilic and hence able to distribute differently from unmodified phosphodiester oligonucleotides (C-5 propyne modification; White *et al*, 1999). In this study we used unmodified phosphodiester oligonucleotide in a cream formulation that appears to enhance the penetration of ODN through stratum corneum and achieve therapeutic concentrations in epidermis and dermis after topical application.

Topical treatment of psoriasis and other inflammatory skin disease is a potential therapeutic application of ICAM inhibition in skin. The results above clearly show the ability of the topical formulation to deliver ODN rapidly to the intended targets within the skin and cause a rapid, complete, concentration-dependent, and potentially durable inhibition of target protein.

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